IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Examiner	: Anne R. Kubelik
Serial No.	: 09/807,723
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Inventors	: Henry Daniell
Title	: GENETIC ENGINEERING OF PLANT CELLS TO PROVIDE ENHANCED EXPRESSION OF MULTIPLE FOREIGN GENES IN TRANSGENIC PLASTIDS UTILITIZING A SINGLE TRANSFORMATION EVENT

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DECLARATION OF HENRY DANIELL

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Professor Henry Daniell, declare that I reside at 1440 Pelican Bay Trail, Winter Park Florida, 32792, and that I am one of the inventors named in Application No. 09/807,723, and I am thoroughly familiar with the technology relating to plastid transformation of higher plants since1977. I am currently a Professor and Trustee Chair at the University of Central Florida and I have served on the faculty of University of Illinois, Washington State University, University of Idaho, Auburn University and the University of Central Florida since 1980 and published over 100 research articles in scientific journals, including invited reviews in the most cited journals in Biotechnology, Molecular Biology and Plant Biology on the transformation of plant plastids. I am familiar with U.S. Serial No. 09/807,723, which has now received an Office Action rejecting Claims 1 and 60 as being anticipated by International Publication No. WO 99/05265.

I have carefully reviewed and confirmed that the subject matter contained in International Publication No. WO 99/05265 lacked enablement at the time of filing and in follow up investigations. Professor Ana M. Bailey, one of the primary inventors of this publication, joined my laboratory as a visiting scientist and continued this investigation because further prosecution

of this patent was halted due to lack of enablement. Please find attached an abstract entitled "Expression of glpA/B operon in transgenic chloroplasts to degrade glyphosate" by Drs. Amit Dhingra, Ana M. Bailey and Henry Daniell, submitted to the 10th International Association for Plant Tissue Culture & biotechnology, June 23-28, 2002. Because of lack of enablement, even with improved chloroplast vectors in my laboratory, this abstract was withdrawn and no presentation was made at this meeting. Laboratory note books containing results of these investigations (inability to express glp A/B operon in transgenic chloroplasts) by Professor Ana Bailey and Dr. Amit Dhingra are available for confirmation of aforementioned statement.

I have attached herewith Exhibit A (Abstract published at the 10th IAPTC meeting), which illustrate the optimism in expressing the constructs disclosed in WO 99/05265 or their derivatives to undergo successful transformation. Specifically, this exhibit shows further improvements (using UTRs to further enhance translation) made to express glpA/B operon in transgenic tobacco chloroplasts. Unfortunately, such efforts were also unsuccessful.

The undersigned declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and thus such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: July 7, 2003

H Daniell

Dr. Henry Daniell
Professor & Trustee Chair

P-1004

Platform Technology for Engineering Pest Resistance in Vegetable Crucifers. Huaiyu Wang and DANIEL C. W. BROWN. Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, 1391 Sandford Street, London, Ontario, Canada N5V 4T3; E-mail: browndc@em.agr.ca

Cabbage (*Brassica oleracea var. capitata*) is reputed to be recalcitrant to genetic transformation. We have developed a 3-step protocol optimization approach for *in vitro*-based research that allows a rapid and simultaneous optimization of multiple germplasm accessions. The approach was applied to the optimization of cabbage hybrid and inbred lines and subsequent transformation protocols. Although regeneration capacities of 20 target cabbage lines were low and differed widely in the first *in vitro* screening, the most influential factors affecting regeneration were identified and ranked. A second optimization focused on the four most important factors and regeneration frequencies of most cabbage lines were greatly improved: 75% the lines could be regenerated at a 70%+ regeneration frequency with 10+ shoots per hypocotyl explant. The poorest responding germplasm was subjected to a third optimization round and regeneration frequency was increased to 95% with 9.5 shoots per explant. A histological study on organ regeneration from cabbage hypocotyl explants showed the source of *de novo* shoot meristems is the cells adjacent to the vascular bundle with cell divisions found only 8 days after preculture. Optimization of the *Agrobacterium* inoculation method and timing resulted in the successful insertion and expression of a *cry*1A*c* gene. Marker gene expression in different parts of transgenic cabbage plants was quantified by ELISA and bioassays with target insect larvae resulted in 100% mortality without perceivable leaf damage.

P-1005

Gene Profiling on Blighted and Healthy Citrus Plants. E. F. CARLOS[1], K. S. Derrick[2], G. Barthe[2], and G. A. Moore[1]. [1]Horticultural Science, University of Florida, Gainesville, FL and [2]CREC, University of Florida, Lake Alfred, FL. E-mail: ECARLOS@UFL.EDU

Citrus Blight is an important citrus disease affecting primarily yield in adult plants and compromising maintenance of entire commercial blocks. It is present in most citrus producing areas around the world except those with a Mediterranean climate. Blight is associated with rootstock choice and annual tree losses in Florida (USA) and São Paulo (Brazil) range normally from 4 to 6%, making Citrus Blight a very important production concern in both areas. At present the disease is of unknown etiology. Therefore, a cDNA subtractive method was used to study gene expression patterns in blighted and healthy plants. Roots of a blighted Rough Lemon (*Citrus jambhiri* Lush.) rootstock supporting a Valencia sweet orange (*Citrus sinensis* L. Osbeck) canopy were dug out of a sandy soil after the presence of the disease was diagnosed by typical visual symptoms, zinc accumulation in the trunk and immunoassays of a diagnostic protein. Samples from healthy plants were taken from the same block of trees. Total RNA was obtained and RT-PCR was performed using Clontech Smart cDNA procedures, which enrich for messenger RNA transcripts. Hybridizations were performed between split sets of cDNAs of healthy plants against blighted ones, and vice-versa using Clontech PCR-Select cDNA subtractive procedures. Differentially enriched cDNAs were cloned and probed on membrane arrays with P32 labeled cDNAs made from subtracted healthy and blighted samples. Selected clones from 'Healthy' and 'Blighted' libraries were sequenced and analyzed. The process was repeated twice, and among the results, sequences with high homology to metallothionein, putative transporters and chitinase genes were found, as were some of unknown function. The metallothionein result is interesting because Blight causes unbalance in the mineral status of affected plants, and this gene maybe involved in this process. Putative transporters were also interesting because they may be related to the stress that Blight causes in plants. Finally, chitinases may be involved in some plant defense mechanism. Northern analyses to further characterize expression of interesting genes are underway.

P-1006

Expression of glpA/B Operon in Transgenic Chloroplasts to Degrade Glyphosate. AMIT DHINGRA, Ana M. Bailey, and Henry Daniell. Department of Molecular Biology and Microbiology, University of Central Florida, 12722 Research Parkway, Orlando, FL 32826-3227. Email: daniell@mail.ucf.edu

Glyphosate is an effective non-selective herbicide that is completely degraded by soil microorganisms. Several plant species have been engineered for glyphosate tolerance by overexpression of the EPSPS gene via nuclear and chloroplast genomes. In *E. coli* it has been demonstrated that the expression of *glp*A/B operon enables the bacteria to withstand very high levels of glyphosate in the media (10.2 g/liter). The *glp*A gene encodes for a phosphotransferase, which phopsphorylates the herbicide and the second gene *glp*B cleaves it to form AMPA, a substrate metabolized by the C-P lyase. It has been well established in our lab that chloroplast genomes are ideal for single step multigene engineering without the drawbacks of gene silencing and position effects. Therefore in the present study an attempt has been made to express *glp*A/B operon in transgenic chloroplasts under the regulation of specific untranslated regions. Chloroplast integration of the transgene will be confirmed by PCR and Southern analyses. We expect to observe high-level tolerance against glyphosate in the transgenic plants. Due to maternal inheritance of the chloroplast genomes, concern of pollen mediated transgene dissemination will be minimized. An attempt would also be made to assess the potential of the *glp*A/B operon as a selectable marker for chloroplast transformation in tobacco. If successful, it will be employed for the transformation of other cereal crops in the future where the application of this technology is limited due to lack of appropriate selectable markers, besides other limiting factors.

P-1007

Genetically Engineered Resistance for Barley Yellow Dwarf Virus (BYDV) Resistance in Wheat. P. DUPRE(1–2), M. Henry(1), A. Pellegrineschi(1), M. Trottet(2) and E. Jacquot(2). (1) International Maize and Wheat Improvement Center (CIMMYT), Apdo. Postal 6–641, 06600 Mexico D.F. (2) INRA Station de Pathologie Végétale, BP29, 35650 Le Rheu- France.

BYDV-PAV represents one of the most serious viruses of wheat and causes substantial losses throughout the world. In attempt to obtain BYDV resistant wheat, we genetically engineered this specie using the pathogen-derived resistance strategy. A total number of seven constructs harboring the BYDV-PAV sequences encoding for replicase, coat-protein, movement protein or a non-coding sequence corresponding to the promoter of sub-genomic 2 RNA were obtained. These sequences have been cloned into the unique *Bam*HI site of the $pAHC_{17}$ plasmid, in sense and in anti-sense orientation, between the UBI promotor and *Nos* terminator. Three wheat varieties were transformed by microprojectile bombardment with the constructs and the *bar* gene according to a CIMMYT protocol. Variable numbers of transgenic wheat were obtained for each construct–variety combination. For all transgenic plants, the number of copies and number of integration sites were obtained by Southern-blot on the T0 generation. BYDV resistance testing and transgene expression studies are currently being conducted on the T1 generation.